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Preferential Localization of Varying Forms of Photoactive 1,8-Naphthalimide Compounds Within the Atheromatous Arterial Wall

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Background and Objective: **We are currently working with a novel class of photoactivated 4-amino substituted 1,8-naphthalimide compounds for tissue bonding. With promising results in other tissues, we are pursuing potential vascular applications. This study focused on determining the appropriate compound formulation(s), concentration, and exposure times to optimize penetration of the heterogeneous arterial wall.**
Study Design/Materials and Methods: **Segments of atheromatous rabbit carotid artery were immersed in hydrophilic or lipophilic forms of the compound, then frozen, cryosectioned, and examined by confocal microscopy.**
Results: **The hydrophilic compound exhibited preferential localization within the intima and media and limited presence in the adventitia. Conversely, the lipophilic compound concentrated in the intima and adventitia with virtual exclusion from the media. Exposure to both forms resulted in complete penetration of the arterial wall.**
Conclusion: **These results extend our knowledge and permit a more practical approach to potential vascular applications using these photoactivated compounds for tissue bonding. Lasers Surg. Med. 26:316–322, 2000.** **© 2000 Wiley-Liss, Inc.**

Key words: hydrophilic; lipophilic; surgical adhesives; tissue bonding; tissue sealants

INTRODUCTION

Tissue bonding is an exciting and advancing field of medical research. Several compounds are currently under investigation as surgical adhesives and tissue sealants. Despite evidence of their potential, compromising issues remain with each. We are currently working with a newly designed and synthesized class of photoactivated 4-amino substituted 1,8-naphthalimide compounds [1,2]. These compounds have been used to achieve impressive bond strengths in several tissue types. For example, a lipophilic form of the compound (diEd66Br) has been used to bond porcine dura mater (425 g/cm^2) [3]. A modified hydrophilic form of the compound (MBM Gold BW-012-011-012) has been used to achieve tissue bonds in meniscal and articular cartilage with tensile shear strengths of up to 2 kg/cm^2 [4]. Furthermore, bonding by both lipophilic and hydrophilic

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agents occurs in the virtual absence of heat generation with ultrastructural studies verifying the absence of thermal denaturation of protein or tissue coagulation [3]. In vivo studies in a sheep model demonstrate primary healing responses without inflammation or excessive cellular proliferation [4].

These successes and the apparent adaptability of this family of tissue bonding compounds has prompted the investigation of these compounds for tissue bonding in vascular repair. The heterogeneous nature of the arterial wall presents a unique challenge for uniform distribution of the compounds. Therefore, initial experiments focused on determining the appropriate compound formulation(s), concentration, and exposure times to optimize vascular penetration.

MATERIALS AND METHODS

Atherosclerotic lesions were created in the common carotid arteries of young adult (3–6 months), male New Zealand White rabbits (n = 5) by using the air-desiccation model [5]. Approximately 4-cm lengths of both the right and left common carotid artery were harvested and opened longitudinally. Each artery was then divided into 8 small rectangles, providing 16 test segments from each animal. Test segments were immersed into "high" (0.9 mM) or "low" (0.45 mM) concentrations of lipophilic or hydrophilic forms of the compound or vehicle for 5, 15, or 30 minutes. The lipophilic form was dissolved in Cremophor EL®, a micellar agent obtained from BASF Corp., and diluted with phosphate buffered saline (PBS) to provide an aqueous stock solution ("high" = 0.9 mM in 20% Cremophor EL®) and a corresponding control without compound. A 0.9 mM stock solution of the hydrophilic form of the compound was prepared in PBS. "Low" concentrations for each form were made by making a 1:2 dilution of the respective stock solution (0.45 mM). The concentrations used in these experiments permitted the use of confocal microscopy to discern localization without excessive fluorescence.

After compound exposure, tissues were frozen in liquid nitrogen, cryosectioned, and examined by confocal microscopy (λ_{ex} 485 nm, λ_{emit} 520 nm). This procedure permitted qualitative evaluation of compound localization and quantification of compound penetration. Forty frames were acquired and summed for all samples and treatments to achieve a suitable intensity for quantification. A 530-nm filter was used to screen out autofluorescence (more toward the green range) from compound fluorescence (predominantly in the yellow range).

Confocal microscopy images were analyzed by using image analysis software (Global Lab Image by Data Translation, Inc.). The line profile tool was used to measure the band of fluorescence moving in from the luminal side. Multiple line profiles across the arterial wall were averaged to determine the area under the curve and the distance of compound penetration. The histogram tool was used to determine an average intensity of the adventitial tissue fluorescence after exposure to the lipophilic form (Fig. 1).

Statistics

Arterial segment treatment was determined randomly, and all data were tested for normality and homogeneity of variance with the Shapiro-Wilk *W* statistic [6] and the Bartlett Chi Square test [7], respectively. Each animal provided sufficient tissue to contribute to all experimental groups; therefore, a paired statistical design could be used. Because of the heterogeneity of variance, the Friedman statistic (a nonparametric equivalent to the repeated measures analysis of variance) was used, followed by a modified Student-Newman-Keul's multiple comparison test. Significance was defined to be $P < 0.05$ and data are presented as means ± standard deviation.

RESULTS

Immersion of the atherosclerotic tissue in the hydrophilic form resulted in compound penetration from the luminal side at all concentrations and exposure times tested. Dose- and time-dependent responses were demonstrated by increasing penetration with higher doses and longer exposure times (Fig. 2). The area under the curve and the diffusion distance doubled between the 5- and 15-minute exposures. Between 15- and 30-minute exposures, these parameters nearly doubled again. The degree of penetration with the high concentration for 30 minutes was significantly greater than that observed with either concentration at 5 minutes. Considering an average medial thickness of the carotid arteries studied, the average depth of compound penetration may be expressed as a percentage of the medial thickness. After 5 minutes of exposure, the low concentration had penetrated approximately 31% of the medial thickness compared with 41% with the high concentration. After 15 minutes, the low and

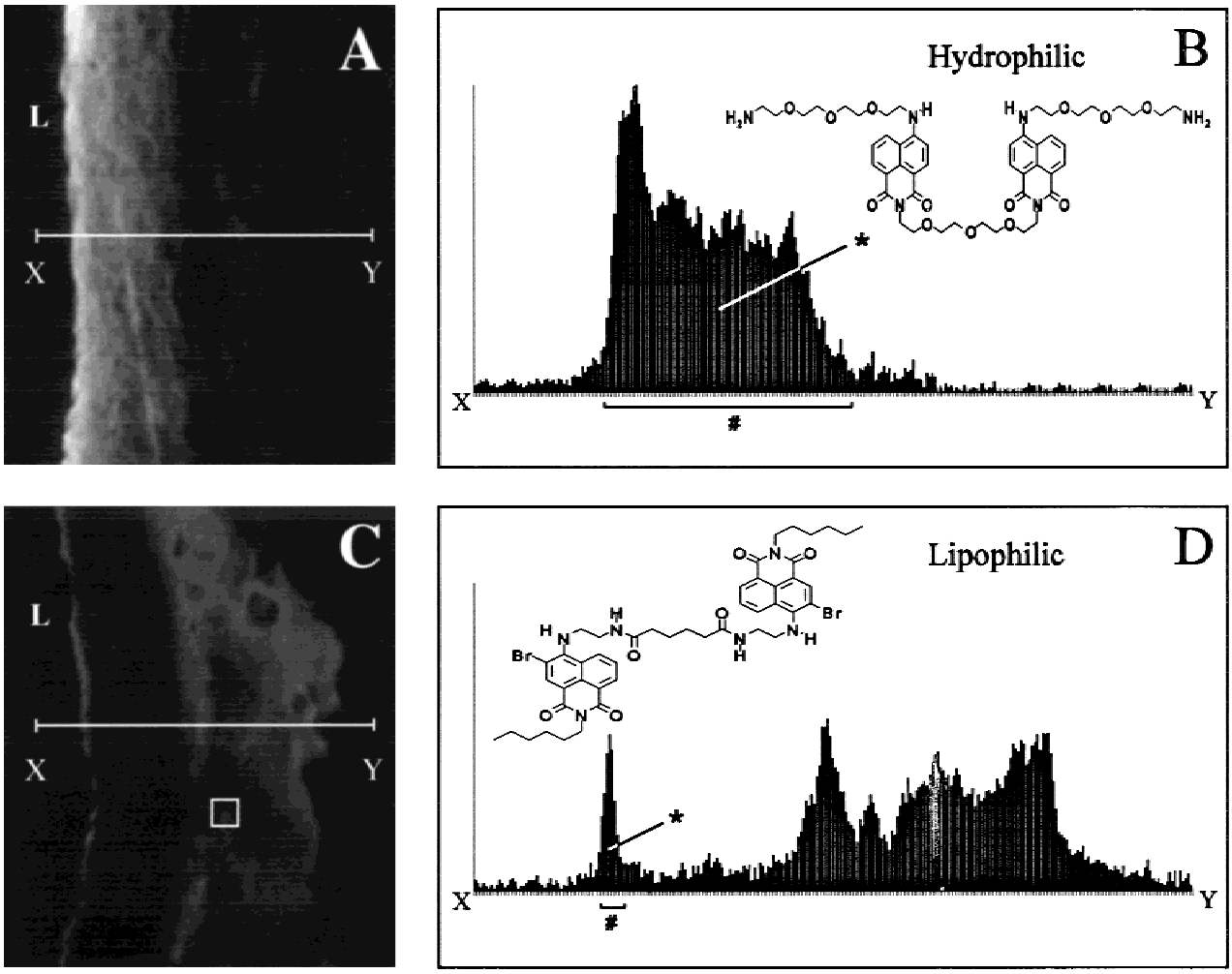

Fig. 1. Confocal microscopy was used to evaluate compound penetration. A line profile of fluorescence was used to evaluate medial fluorescence, which was extensive with the hydrophilic form (**A**) and more limited with the lipophilic form (**C**). **B,D:** Typical line profiles for each respective form by plotting pixel intensity across the line X-Y. A summation of the pixel intensity under the peak was used to calculate area under the curve (asterisks), and the width of the peak determined diffusion distance (pound signs). Adventitial fluorescence (observed only with the lipophilic form) was assessed by averaging the intensity within randomly placed boxes of equal size (C). L, lumen.

high concentrations penetrated 54% and 80%, respectively. After 30 minutes of immersion, the low concentration penetrated 77% of the media, whereas the high concentration spanned the entire media and a portion of the adventitia on the medial side (129%).

Immersion of atherosclerotic tissue into the lipophilic form also resulted in tissue penetration, but in contrast to the hydrophilic form, there was very limited passage into the media but substantial uptake by the adventitia (Fig. 3). Again, the concentration and exposure time influenced the degree of penetration. With the low concentration, penetration increased by about 50% between 5 and 15 minutes and an additional 30% between 15 and 30 minutes. With the high concentration, penetration was 3.5 times greater at 15 than at 5 minutes. Between the 15- and 30-minute exposures, penetration increased another 20%. Use of the Friedman statistic revealed a statistical difference between groups. However, this difference was not detectable by the modified Student-Newman-Keuls test used for multiple comparisons.

The previous analyses have considered the penetration of each compound form independently. Although both the hydrophilic and lipophilic forms showed a capability to penetrate from the luminal side, the magnitude was very different. The graphs in Figure 4 compare the relative

degree of penetration for each experimental group. Neither of the control groups demonstrated fluorescence beyond that attributable to autofluorescence. Lipophilic compound penetration, even at high concentrations for 30 minutes, was significantly less than that observed with hydrophilic compound.

DISCUSSION

Previous evaluations of these naphthalimide compounds have been in relatively homogeneous tissue types with rather uniform and predictable compound penetration. In the arterial wall, lipophilic and hydrophilic forms of the compound revealed unique penetration patterns. These patterns likely reflect the heterogenous, multilayered structural nature of the arterial wall and can be explained in part by considering the constituents of the various wall components.

The dense irregular connective tissue of the adventitia showed an affinity for the lipophilic form similar to that observed previously in porcine dura mater [3]. Furthermore, adventitial adipose cells may enhance adventitial penetration. These animals had been on a high cholesterol diet for a month before tissue harvest, which could contribute to adipose deposition. The lipid-loading of intimal cells would serve as a suitable substrate for lipophilic compound penetration and localization did occur here. Oil red O staining of representative arterial cross-sections revealed that specimens with substantial plaque development and intimal accumulation of lipids also had enhanced adventitial adipose deposition. However, although there was a high degree of variability in lipid accumulation, there was a consistent localization of the lipophilic form in both the intima and adventitia. This finding suggests that lipid accumulation may augment compound localization but is not essential to the penetration of these constituents.

Of particular interest in these specimens was the consistent absence of the lipophilic compound in the media at all concentrations and times tested. Lipophilic compound penetration seemed to be clearly delimited by the internal and external elastic laminae with virtual exclusion from the media. Although the lipid content of intimal elastin is inherently small (2–3% in nonplaque samples and 4–6% in plaque intima) [8], it has been suggested that the hydrophobicity of elastin proteins may play an important part in the binding of lipoproteins to arterial elastin [9]. These data provide conflicting rationale for the repulsion or attraction of a lipophilic substance. This situation is further complicated by the findings of Velebny et al. [10] that suggest the interaction of alpha-elastin with a compound depends more on its chemical structure than on its charge, and that interaction mechanisms differ for varying concentrations of the same compound. Regardless, it seems that either the composition of the elastic laminae or the composition of the media itself served as a barrier for diffusion of the lipophilic form.

Diffusion patterns of the hydrophilic compound were consistent, yet surprising. Considering that the hydrophilic form was designed to penetrate connective tissue, its absence in the adventitia was notable. The structural and compositional differences in the matrices of specialized connective tissue (as in the case of cartilage) and dense connective tissue (dura mater and adventitia) may contribute to the affiliation for hydrophilic or lipophilic substances. Adventitial lipid deposition within adipose cells would further restrict the movement of hydrophilic compound into the adventitial side. Theoretically, the presence of a lipid-laden plaque may serve as a deterrent for luminal entry. However, luminal entry was readily apparent in all specimens examined. It may be that the hydrophilic form is allowed luminal access for diffusion in diseased arteries by means of the associated extracellular matrix and other components of the plaque with an affinity for water-soluble materials.

Additional Testing

Recent improvements in the synthesis and purification of these naphthalimide compounds has allowed for testing of much higher concentrations. Additionally, sequential exposures to both hydrophilic and lipophilic forms of the compound were conducted to determine whether the entire arterial wall could be penetrated. Normal carotid arterial specimens were subjected to a 15-minute exposure to 15 mM hydrophilic compound followed by a 15-minute exposure to 5 mM lipophilic compound. This protocol clearly resulted in compound penetration of all arterial wall components, and fluorescence intensity far exceeded that observed previously (Fig. 4C). When confocal microscopy was conducted as previously described, only a single frame was required (summation of two frames resulted in offscale fluorescence). For

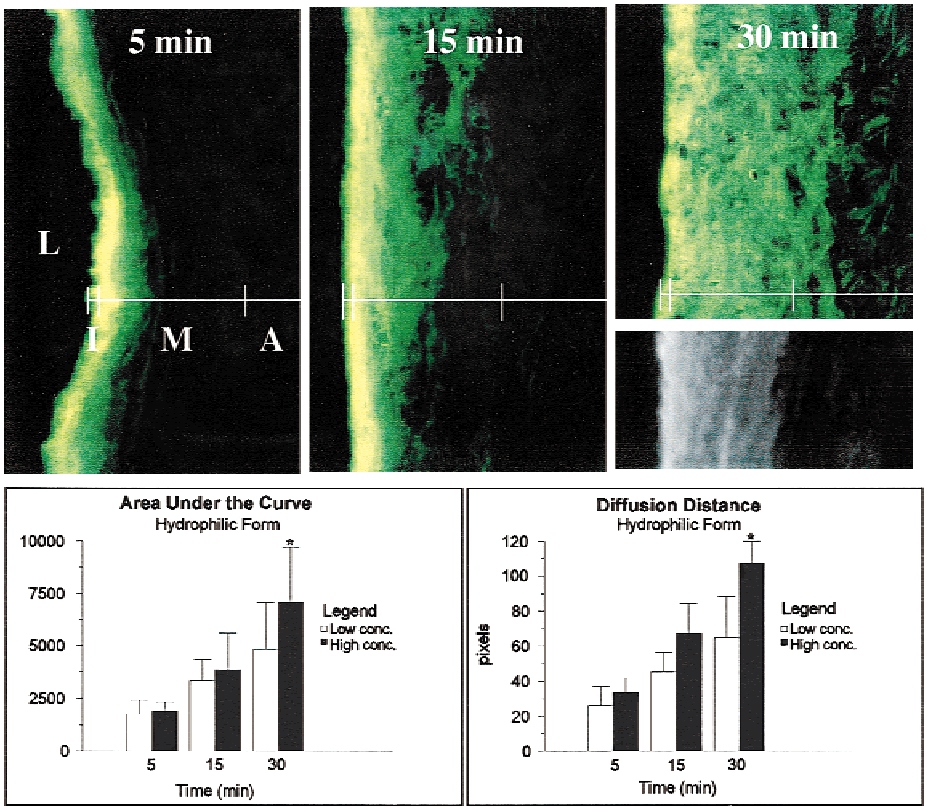

Figure 2

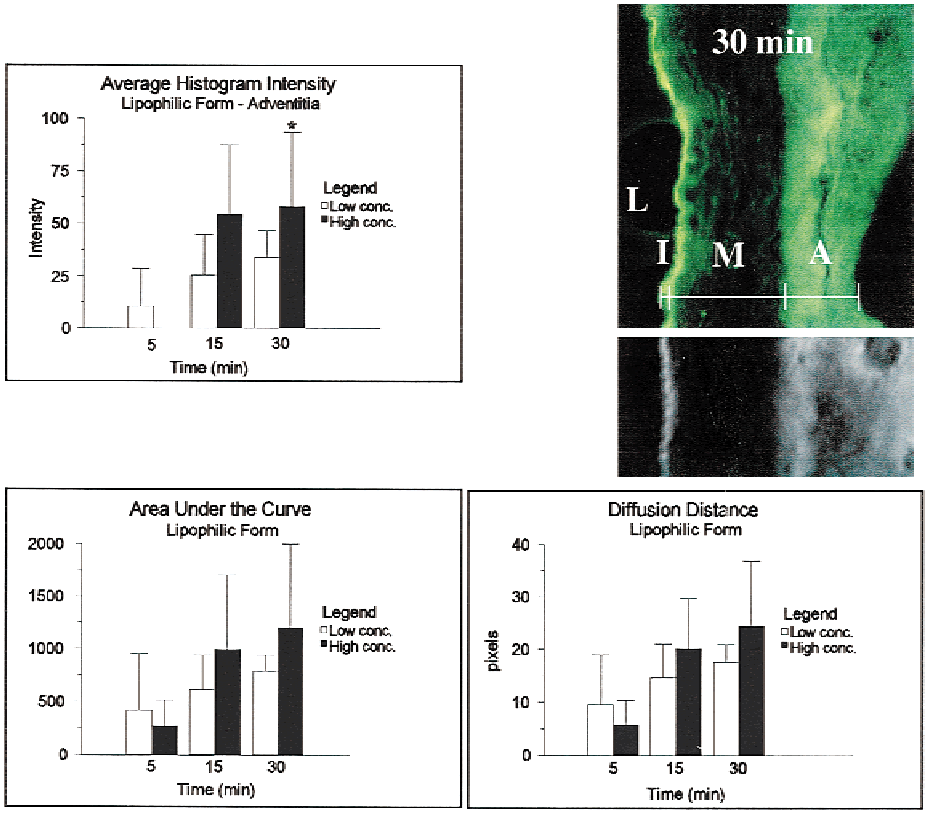

Figure 3

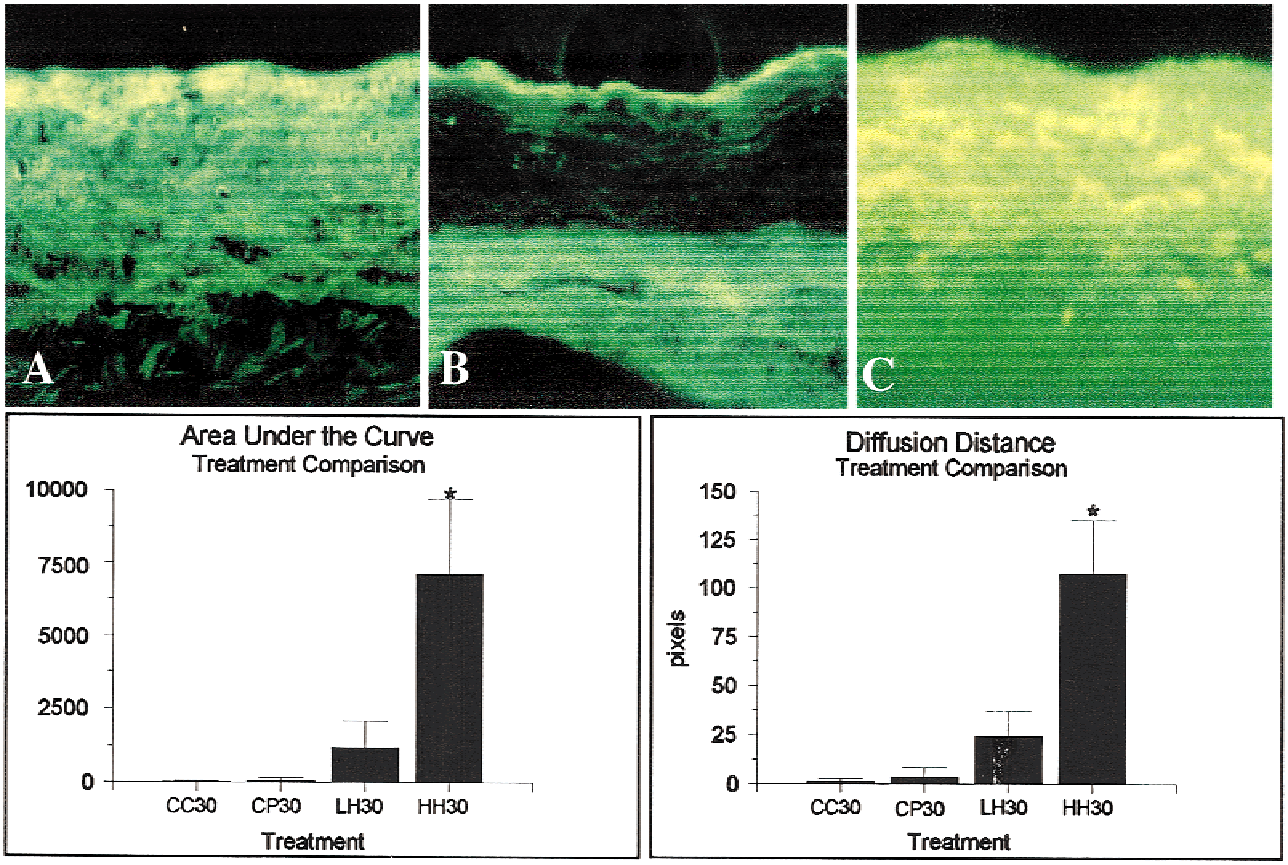

Fig. 4. The top panels provide a visual comparison of the compound penetration patterns of the hydrophilic (**A**) and the lipophilic (**B**) forms of the compound. The graphs show the comparative degree of compound penetration between the initial treatments. **C:** The use of higher concentrations and a combined exposure to both the hydrophilic and lipophilic forms resulted in complete penetration of the arterial wall and enhanced brilliance. Asterisks indicate $P < 0.05$ compared with all other groups; n = 5; CC30, control with Cremophor for 30 minutes; CP30, control with phosphate buffered saline for 30 minutes; LH30, lipophilic compound, high dose, for 30 minutes; HH30, hydrophilic compound, high dose, for 30 minutes.

this reason, direct comparison could not be made between these and previous specimens (summation of 40 frames).

Perspectives

These studies have provided the data to more knowledgeably approach potential vascular applications using these photoactivated compounds for tissue bonding. The targeted component of the arterial wall will determine the single compound form or combined exposure most suited for each application. For example, these results suggest that tissue bonding within the adventitia may be most successfully achieved by using a lipophilic form, whereas, hydrophilic forms may be most suited for medial components. For tissue

Fig. 2. The top panels illustrate the penetration of the hydrophilic form of the compound from the luminal side over time. L, lumen; I, intima; M, media; A, adventitia. The insert on the lower portion of the 30 minute panel shows a corresponding filtered confocal microscopic image. Note the loss of some fluorescence believed to be predominantly autofluorescence. The graphs illustrate the proportionate penetration of the compound as a function of area under the curve or diffusion distance. Asterisks indicate $P < 0.05$ compared with either concentration at 5 minutes; n = 5.

Fig. 3. The upper right panel illustrates typical penetration of the lipophilic form with minimal entry from the luminal side, virtual absence in the media, and significant adventitial fluorescence. L, lumen; I, intima; M, media; A, adventitia. The inset shows a corresponding confocal microscopic image. Note the filtering of medial autofluorescence. The top graph shows the magnitude of adventitial fluorescence over time. The lower graphs illustrate the proportionate penetration of the compound from the luminal side as a function of area under the curve or diffusion distance. Asterisks indicate $P < 0.05$ compared with high concentration for 5 minutes; n = 5.

bonding across the entire arterial wall, as perhaps with vascular anastomoses, exposure to both forms may achieve the strongest bonds. In vitro tests to determine the ability of these compounds to bond vascular tissue are currently under way in our laboratory, and subsequent testing will be required to determine their effectiveness in vivo.

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